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(54) Title: MONOCLONAL ANTIBODY TO HUMAN TYPE IX COLLAGEN

(57) Abstract

Oligopeptides corresponding to segments of human Type IX collagen are useful as antigens in producing monoclonal antibodies which bind immunologically to human Type IX collagen and fragments derived from it and which can be used to detect their presence in biological fluids such as synovial fluid and serum.

LEDIGLICH ZUR INFORMATION

Code, die zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

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MONOCLONAL ANTIBODY TO HUMAN TYPE IX COLLAGEN

This invention relates to purified oligopeptides corresponding to fragments of human Type IX collagen and to monoclonal antibodies which bind specifically to human Type IX collagen.

Type IX collagen is one of a distinct class of extracellular matrix proteins associated with the surface of collagen fibrils in cartilage. In patients suffering from degenerative cartilage diseases such as rheumatoid arthritis and osteoarthritis, the Type IX collagen is disrupted, so that it or fragments of it are no longer in their normal location and can instead be found in the intra-joint fluid, for example. This abnormal condition of Type IX collagen is symptomatic of the disease.

A number of earlier publications describe the structure and composition of chicken Type IX collagen, such as Konomi, et al., J. Biol. Chemistry Vol. 261, 6742-6746 (1986); McCormick, et al., Proc. Natl. Acad. Sci. USA Vol. 84, 4044-4048 (1987); Vasios, et al., J. Biol. Chemistry Vol. 263, 2324-2329 (1988); Svoboda, et al., Proc. Natl. Acad. Sci. USA Vol. 85, 7496-7500 (1988); Olsen, et al, Anal. of NY Acad. Sci., Vol 460,pp 141-153 (1985); Ninomiya, et al., Proc. Natl. Acad. Sci. USA, Vol. 81, pp 3014-3018, (1984); Lozano, et al., Proc. Natl. Acad. Sci. USA Vol. 82, pp.4050-4054 (1985); Ninomiya, et al., Biochemistry, Vol. 24, pp. 4223-4229, (1985); van der Rest, et al., J. Biol. Chemistry, Vol 260, pp 220-225 (1985); Huber, et al., J. Biol.

Chemistry, Vol. 261, pp. 5965-5968 (1986); Konomi, et al., J. Biol. Chemistry, Vol 261. PP. 6742-6746 (1986); Vaughan, et al., J. Cell Biol., Vol 106, pp. 991-997 (1988); Snyderman, et al., Arthritis and Rheumatism, Vol. 30, pp 1191-1194 (1987); McCormick, et al., Proc. Natl. Acad. Sci. USA, Vol. 84, pp. 4044-4048, (1987); Vasios, et al., J. Biol Chemistry, Vol. 263, pp. 2324-2329, (1988); Svoboda, et al., Proc. Natl. Acad. Sci. USA, Vol 85, pp. 7496-7500, (1988); Olsen, et al., Glauert (ed), The Control of Tissue Damage, pp 29-39, (1988); Olsen, Biol. Basis of the Human Chondrodysplasias, Pathol. Immunopathol. Res. 7: 20-23 (1988). Also, it has been demonstrated that human cartilage contains a similar molecular structure (Bruckner, et al., J. Biol. Chem., Vol 263, 16911-16917 (1988). However, human Type IX collagen differs in amino acid sequence and composition from chicken Type IX collagen to a significant extent; for example, probes containing cDNAs for chicken Type IX collagen do not cross hybridize well with human genomic DNA for the human Type IX collagen.

We have now cloned several cDNAs that collectively encode the α1 (IX) chain of human Type IX collagen, including its amino-terminal globular domain (NC4), thus determining the amino acid sequence of the protein chain encoded by the DNA, have synthesized antigenic oligopeptides corresponding to fragments of the protein, and have prepared against these antigens monoclonal antibodies which bind specifically to human Type IX collagen, or human α1 (IX) collagen chains or fragments thereof. The antibodies can be labeled by conventional procedures with radioactive, fluorescent, enzyme or other labels and used in a procedure for assaying biological fluids such as synovial fluid for

detecting the presence or absence of Type IX collagen, α1 (IX) collagen chains or fragments thereof, thus serving as a diagnostic tool for the presence or absence of a degenerative cartilage disease. In addition, the antibodies can be used to screen drugs for use against such disease.

Figs 1A and 1B show the amino acid sequence of the human α1 (IX) collagen chain as deduced from the cloned cDNAs.

The cDNAs coding for the protein sequence of Fig. 1 were obtained by conventional extraction and isolation of RNA from chondrocytes derived from a costal cartilage specimen obtained from a female patient, followed by synthesis and cloning of the cDNA according to procedures well known in the art. The human cDNA library was screened by filter hybridization, positive phages purified, and recombinant DNA isolated using standard procedures, and nucleotide sequence analysis was performed by the Maxam and Gilbert procedure as well as by the dideoxy chain termination technique.

From the sequence of Fig. 1 the following segments were selected as containing the most promising epitopes:

Example 1

Gly-Arg-Ala-Pro-Thr-Asp-Gln-
His-Ile-Lys-Gln-Val-

Example 2

Glu-His-Phe-Ala-Glu-Met-Ala-Ala-Ser-Leu-
Lys-Arg-Pro-Asp-Ser-Gly-Ala-Thr

The oligopeptides of Examples 1 and 2 (oligopeptide 1 and oligopeptide 2) were then synthesized by conventional Merrifield solid phase synthesis, and there was added to the carboxyl end of each a cysteinyl residue to enable the oligopeptide to be coupled to a carrier protein. After purification, each of the synthetic peptides was coupled through the carboxy-terminal cysteinyl residue to keyhole limpet hemocyanin by conventional procedures. Lyophilized peptide (5 mg) was coupled to 4 mg of hemocyanin (Polysciences) in about 2 ml of 20 mM sodium phosphate-buffer pH 7.5. After incubation at room temperature for 3 hours, peptide-hemocyanin complexes were separated from uncoupled peptide by gel filtration on Sephadex G-50 (fine) (1x25cm) equilibrated with 20 mM sodium phosphate pH 7.5.

For generation of antibodies, 50-100 µg of peptide-hemocyanin complex was mixed in 0.5 ml of complete Freunds Adjuvant and injected intraperitoneally into Balb-C mice. Booster injections were given at two week intervals in incomplete Freunds Adjuvant. A total of 2-4 booster injections were given. Mice were periodically bled from the tail vein for screening of sera. When sera were positive in ELISA assays against peptide-BSA conjugates, subcutaneous injections of 10-20µg without adjuvant were given twice, one 5 days before fusion and the second 3 days before fusion. Mouse splenocytes were fused with myeloma cells (NS1-Ag4/1) using standard methodologies (Kohler and Milstein, Nature Vol. 256, 495 (1975)), and the resulting hybridomas were cultured and screened for specificity to their respective polypeptide antigens.

Hybridoma supernatants were screened by ELISA assay. Immunofluorescence was performed on cryostat sections, which had previously been digested with testicular hyaluronidase (3.3 mg/ml in phosphate buffer pH 6.0) for 0.5 hour at 37°C. Primary antibody incubations were for 1 hour at room temperature. Following washes (3x5 min), the secondary antibody was applied (fluorescein-goat anti-mouse IgG). Culture supernatants from non-antibody producing hybridomas were collected and utilized as negative control for primary antisera.

Ten hybridoma strains were selected by such screening for each of the Example 1 and Example 2 oligopeptides. A specimen of the culture of each of the ten strains was injected into a pristane-primed mouse, the ascites collected, and the monoclonal antibody purified from the ascites by precipitation in 40% ammonium sulfate, then chromatographed on a column containing DEAE Sephacel. The antibodies were finally subjected to electrophoresis on SDS-polyacrylamide gels for assessment of purity. All monoclonal antibodies were determined to be of the IgG class. They were of various subclasses and types as follows:

IgG_1/κ;
IgG_{2A}/κ;
IgG_{2B}/κ;

All of the ten purified monoclonal antibodies against each of Examples 1 and 2 were tested for immunoreactivity with human $\alpha 1$ (IX) collagen chains by the following procedure. Specimens of costal human cartilage were frozen in liquid nitrogen, were smashed to

a powder and lyophilized. The powder was then treated overnight in a suitable buffer with the enzyme chondroitinase ABC. The mixture was lyophilized and the resulting powder dissolved by boiling in a buffer containing 2% sodium dodecyl sulfate (SDS). This extract was reduced with ß-mercaptoethanol and then subjected to SDS-gel electrophoresis and the separated protein bands in the gel were transferred by Western blotting onto nitrocellulose paper. The individual blots were then treated with a solution of each of the 20 antibodies in phosphate-buffered saline, and with an antibody concentration of 0.005 mg/ml.. A commercial enzyme-labeled second antibody was then employed to determine which of the individual monoclonal antibody specimens reacted with the intact α1(IX) collagen chain on the nitrocellulose blot .

Of the ten monoclonal antibodies against the oligopeptide of Example 1, three reacted strongly: 23-3E12; 23-5D1; 23-2F6. Of the ten antibodies against the oligopeptide of Example 2, one reacted strongly: 24-3G3. A specimen of hybridoma line 24-3G3 was deposited with the American Type Culture Collection under the Budapest Treaty on January 13, 1989, being identified as No. HB 9972.

These results were corroborated and extended by subjecting each of the four immunoreactive monoclonal antibodies to a standard immunofluorescent assay for binding to Type IX collagen in a specimen of human articular cartilage. Antibodies 23-3E12, 23-5D1, and 23-2F6 were also found to bind to specimens of mouse and rat Type IX collagen, but not to chicken Type IX collagen.

Oligopeptides containing the following sequences

from within the amino-terminal globular portion of the human α1(IX) chain as shown in Fig. 1 can also be synthesized by conventional solid state synthesis:

Glu-Tyr-Ser-Phe-Leu-Thr-Thr-Phe-
Arg-Met-Thr-Gly

Phe-Arg-Ile-Pro-Thr-Arg-Asn-Leu-Tyr-
Pro-Ser-Gly

In each case the oligopeptide can be coupled to a carrier protein by adding a single cysteinyl moiety to the carboxyl terminus, then using a conventional cross-linking agent. These carrier-supported oligopeptides can then be used as antigens in a manner analogous to that of Examples 1 and 2 to produce monoclonal antibodies.

The monoclonal antibodies immunoreactive with human Type IX collagen and human α(IX) collagen chains can be labeled by any conventional procedure with any suitable label and employed in a conventional assay procedure for detecting the presence or absence of human Type IX collagen, α1(IX) collagen chains or fragments thereof in a specimen of biological fluid such as serum, synovial fluid or the like. The biological fluid to be assayed can assayed by ELISA assays, radioimmunoassays or any other conventional immunoassays using the monoclonal antibodies.

Claims

1. A purified oligopeptide containing the sequence
Gly-Arg-Ala-Pro-Thr-Asp-Gln-His-Ile-Lys-Gln-Val-Cys.

2. A purified oligopeptide containing the sequence
Glu-His-Phe-Ala-Glu-Met-Ala-Ala-Ser-Leu-Lys-Arg-Pro-Asp-Ser-Gly-Ala-Thr-Cys.

3. A monoclonal antibody immunoreactive with human Type α1(IX) collagen chains, or fragments thereof.

4. A monoclonal antibody as claimed in claim 3 obtainable from hybridomas of cell line ATCC No. HB 9972.

5. The method of assaying a composition for human Type α1(IX) collagen chains, or fragments thereof, which comprises providing a monoclonal antibody as claimed in claim 3, labelling said antibody, contacting said composition with said antibody and determining whether or not said antibody binds to said composition.

FIG. 1A

Gly Ala Lys Gly Val Ala Gly Glu Lys Gly Ser Thr Gly Ala Pro
Gly Lys Pro Gly Gln Met Gly Asn Ser Gly Lys Pro Gly Gln Gln
Gly Pro Pro Gly Glu Val Gly Pro Arg Gly Pro Arg Gly Leu Pro
Gly Ser Arg Gly Glu Leu Gly Pro Val Gly Ser Pro Gly Leu Pro
Gly Lys Leu Gly Ser Leu Gly Ser Pro Gly Leu Pro Gly Leu Pro
Gly Pro Pro Gly Leu Pro Gly Met Lys Gly Asp Arg Gly Val Val
Gly Glu Pro Gly Pro Lys Gly Glu Gln Gly Ala Ser Gly Glu Glu
Gly Glu Ala Gly Glu Arg Gly Glu Leu Gly Asp Ile Gly Leu Pro
Gly Pro Lys Gly Ser Ala Gly Asn Pro Gly Glu Pro Gly Leu Arg
Gly Pro Glu Gly Ser Arg Gly Leu Pro Gly Val Glu Gly Pro Arg
Gly Pro Pro Gly Pro Arg Gly Val Gln Gly Glu Gln Gly Ala Thr
Gly Leu Pro Gly Val Gln Gly Pro Pro Gly Arg Ala Pro Thr Asp
Gln His Ile Lys Gln Val Cys Met Arg Val Ile Gln Glu His Phe
Ala Glu Met Ala Ala Ser Leu Lys Arg Pro Asp Ser Gly Ala Thr
Gly Leu Pro Gly Arg Pro Gly Pro Pro Gly Pro Pro Gly Pro Pro
Gly Glu Asn Gly Phe Pro Gly Gln Met * * * * * *
* * * * * * * * * * * * * * *
Gly Asp Leu Gly Glu Lys Gly Glu Arg Gly Pro Pro Gly Arg Gly
Pro Asn Gly Leu Pro Gly Ala Ile Gly Leu Pro Gly Asp Pro Gly
Pro Ala Ser Tyr Gly Lys Asn Gly Arg Asp Gly Glu Arg Gly Pro
Pro Gly Leu Ala Gly * * * * * * * * * *
* * * * * * * * * * * * * * *
* * * * * * * * * * * *

FIG. 1B

Met-Lys-Thr-Cys-Trp-Lys-Ile-Pro-Val-Phe-Phe-Phe-Val-Cys-Ser-
Phe-Leu-Glu-Pro-Trp-Ala-Ser-Ala-Ala-Val-Lys-Arg-Arg-Pro-Arg-
Phe-Pro-Val-Asn-Ser-Asn-Ser-Asn-Gly-Gly-Asn-Glu-Leu-Cys-Pro-
Lys-Ile-Arg-Ile-Gly-Gln-Asp-Asp-Leu-Pro-Gly-Phe-Asp-Leu-Ile-
Ser-Gln-Phe-Gln-Val-Asp-Lys-Ala-Ala-Ser-Arg-Arg-Ala-Ile-Gln-
Arg-Val-Val-Gly-Ser-Ala-Thr-Leu-Gln-Val-Ala-Tyr-Lys-Leu-Gly-
Asn-Asn-Val-Asp-Phe-Arg-Ile-Pro-Thr-Arg-Asn-Leu-Tyr-Pro-Ser-
Gly-Leu-Pro-Gln-Glu-Tyr-Ser-Phe-Leu-Thr-Thr-Phe-Arg-Met-Thr-
Gly-Ser-Thr-Leu-Lys-Lys-Asn-Trp-Asn-Ile-Trp-Gln-Ile-Gln-Asp-
Ser-Ser-Gly-Lys-Glu-Gln-Val-Gly-Ile-Lys-Ile-Asn-Gly-Gln-Thr-
Gln-Ser-Val-Val-Phe-Ser-Tyr-Lys-Gly-Leu-Asp-Gly-Ser-Leu-Gln-
Thr-Ala-Ala-Phe-Ser-Asn-Leu-Ser-Ser-Leu-Phe-Asp-Ser-Gln-Trp-
His-Lys-Ile-Met-Ile-Gly-Val-Glu-Arg-Ser-Ser-Ala-Thr-Leu-Phe-
Val-Asp-Cys-Asn-Arg-Ile-Glu-Ser-Leu-Pro-Ile-Lys-Pro-Arg-Gly-
Phe-Ile-Asp-Ile-Asp-Gly-Phe-Ala-Val-Leu-Gly-Lys-Leu-Ala-Asp-
Asn-Pro-Gln-Val-Ser-Val-Pro-Phe-Glu-Leu-Gln-Trp-Met-Leu-Ile-
His-Cys-Asp-Pro-Leu-Arg-Pro-Arg-Arg-Glu-Thr-Cys-His-Val-Leu-
Pro-Ala-Arg-Ile-Thr-Pro-Ser-Gln-Thr-Thr-Asp-Glu-Arg-

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00283

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5) : C12P 21/02; C12S 3/16
U.S. Cl : 530/327,356

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/327,356

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstracts Services Online (File CA, 1967-1988; File Biosis Previews 1969-1988). Automated Patent System (File US PAT, 1975-1988).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Journal of Biological Chemistry, Volume 263, No. 32, Issued November 1988, P. Bruckner, "The Structure of Human Collagen Type IX and its Organization in Fetal and Infant Cartilage Fibrils", See Pages 16911-16912.	1

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 April 1990	04 MAY 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Laurie A. Scheiner

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [X] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

(SEE CONTINUATION SHEET - Page 4)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

CONTINUATION SHEET - FORM PCT/ISA/210

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

I. Claim 1 is drawn to an Oligopeptide, Classified in Class 530, Subclass 327.

II. Claim 2 is drawn to an Oligopeptide, Classified in Class 530, Subclass 326.

III. Claims 3-5 are drawn to an Antibody and a Method of Use, Classified in Class 435, Subclass 7.

Group I is directed to an Oligopeptide Product while Group II is directed to a separate and distinct Oligopeptide; Group III is directed to a separate and distinct immunological Method. The Oligopeptides of Group I and II could be used in a materially different method than that of Group III such as affinity chromatography. The method of Group III uses a product different from that of Groups I and II. The search burden involved for each additional invention is undue, not only insofar as the additional patent search, but also insofar as considerable Literature searches covering the entire class range noted above.